IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: Frank Grosveld **Confirmation No. 1498**

Serial No.: 10/693,308 **Art Unit: 1632**

Filing Date: October 24, 2003 **Examiner: Anoop K. Singh**

For: IMMUNOGLOBULIN 2

Customer No.: 34132

DECLARATION OF DR. FRANK GROSVELD

1. I am the inventor of the above-identified patent application. I have read the Final Rejection dated as mailed July 9, 2007, and would reply to several the issues raised as follows below.

2. The invention describes methods for the in vivo derivation of heavy chain only antibodies in transgenic non-human mammals in response to antigen challenge. The invention requires a modification to the normal mammalian heavy chain locus such that the CH1 domain is not expressed in the heavy chain following the gene activation as a result of antigen challenge in specialised B-cells. In the absence of a CH1 domain the modified heavy chain cannot combine with light chain even if light chain is present (i.e. in a wild type mouse background) and as a result heavy chain only antibody (devoid of CH1) is secreted and circulates in plasma. B-cell specific expression is necessary for a productive response to antigen stimulation leading to VDJ re-arrangement and, ultimately, secretion of antigen specific heavy chain only antibody (devoid of CH1). This would not occur in other cell types (e.g. skin, muscle, heart)

3. To ensure B-cell specific expression of the transgene, human regulatory elements known to induce B-cell specific expression in non-human mammals are present in the natural human IgH sequence described in the application as filed and used by Janssens et al. At the time of the invention, it had already been established for sometime that the insertion of a human heavy chain locus (comprising the CH1

domain) with a human immunoglobulin light chain locus in a mouse background resulted in the in the production of human antibodies in response to antigen. (See Green et al. *Nat Genet* (1994) 7:13-21, "Antigen specific human monoclonal antibodies from mice engineered with human Ig heavy and light chain YACs"; and Gallo et al. *Eur J. Immunol* (2000) 30:534-540, "The human immunoglobulin loci introduced into mice: V (D) and J gene segment usage similar to that of adult humans", copies enclosed). The feature which differentiates this invention, from the prior art, is the absence of a CH1 region. Thus in the cited references the co-expression of light chain was necessary for the productive expression of a normal H2L2 tetrameric antibody from B-cells(light chain constant region binds to the CH1 domain present in the natural immunoglobulin heavy chain). However, as we show, the expression of a human heavy chain gene constructs devoid of CH1 domains allows the functional expression of heavy chain only dimers alone whether the light chain loci are expressed or not. Thus, the elimination of the CH1 domain from any functional heavy chain gene loci and expression in a non-human mammalian background will result in B-cell specific expression of heavy chain only antibody (devoid of CH1) in response to antigen challenge. This is the essence of the invention as exemplified.

4. Based on the knowledge at the time of filing, we expected the regulatory elements present in the constructs used to drive transgene expression in the B-cells of mammals. The additional presence of IgH LCR regulator elements, while not a requirement of the invention, ensures that every insertional event results in a transcriptionally active IgH (devoid of CH1) transgene in every B-cell.

5. In the Final Rejection, the examiner raises questions as to the genetic backgrounds necessary to enable the invention. As we have shown, the genetic background of the mice used in these experiments is irrelevant. There are **preferred** backgrounds in which the endogenous mouse genes are suppressed or eliminated. For example, we have used the μMT mouse. (*See* Kitamura, D., J. Roes, R. Kühn, K. Rajewsky. 1991. Nature 350:423.) In this strain endogenous mouse immunoglobulin gene expression is blocked early in B-cell development so, whilst mouse IgM is detectable, circulating

levels of mouse immunoglobulin are very low. Figure1 shows a FACS analysis of B-cells from the μMT mouse and reveals the presence of low amounts of mouse IgM.

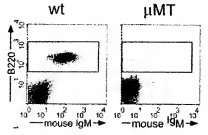


Figure 1 *FACS analysis comparing mouse IgM epression in wild type mice versus μMT mice. The vertical axis shows cell expressing a B cell marker (B220), the horizontal axis cells expressing mouse IgM expression.These panels are copied from Janssens et al 2006, Figure 4A.*

In the absence of significant endogenous mouse IgH gene expression, essentially all plasma immunoglobulin is derived from the introduced heavy chain only transgene. Thus the suppression of endogenous immunoglobulin genes is advantageous, since it facilitates the analysis of heavy chain only gene expression, but is not essential. For example, human immunoglobulin heavy chain only IgM (Figure 2)

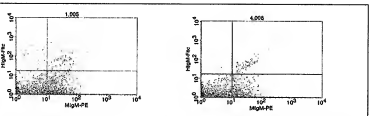


Figure 2 *FACS analysis of heavy chain only IgM expression in a wild type mouse backround.The vertical axis shows cells expressing human IgMfrom the MΔGΔ locus (Janssens et al., 2006), the horizontal axis cells expressing mouse IgM. The panel on the left is a transgenic mouse on a wild type background, the panel on the right a wild type mouse. This figure was not included in Janssens et al 2006*

and human immunoglobulin heavy chain only IgG (Figure 3 copied from Janssens et al 2006) are also expressed from transgenes in normal wild type mouse background.

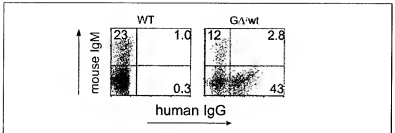


Figure 3 *FACS analysis comparing mouse IgM versus human IgG expression in wild type mice. The vertical axis shows cell expressing mouse IgM, the horizontal axis cells expressing human IgG. The panel on the left is a nontransgenic wildtype mouse, the panel on the right a transgenic mouse on a wild type background. These panels are copied from Janssens et al 2006. Figure 3F.*

To illustrate further the irrelevance of host background to this invention, we enclose details of a human heavy chain only locus where four human V segments replace the two camelid V segments used by Janssens et al. (Fig. 4)

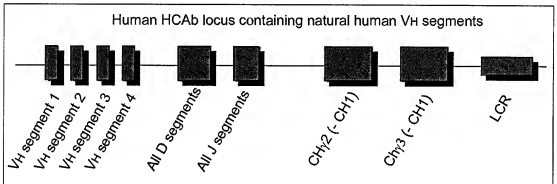


Figure 4 *Scheme of a completely human antibody locus containing natural VH regions. The locus (not to scale) contains the VH3 subclass regions indicated in combination with all of the human D regions, all of the human JH regions, and the human Cγ2 and Cγ3 regions lacking CH1. Lack of the CH1 regions ensures that the locus will result in the production of heavy chain only antibodies.*

This locus is inserted into the chromatin of a wt mouse (FVB strain) where the endogenous murine heavy and light chain genes remain functional. Analysis of serum shows the presence of human heavy chain only antibody (Fig.5 below).

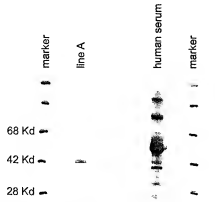


Figure 5 *Western blot showing the expression of human heavy chain only antibody (HAb) in transgenic mice serum containing the 4 natural VH locus (line A) under reducing conditions (i.e. showing single rather than dimer chains although the dimers are visible) . Marker lane shows molecular weight bands, the lane human serum contains normal human IgG. Molecular weights are indicated, the HAb has the predicted size around 40Kd.*

Sequence analysis of human heavy chain only antibody mRNA derived from naïve peripheral B-cells isolated from blood shows that VDJ rearrangement of the transgene occurs as expected in B-cells (Fig.6 below).

EVQLVETGGGLIQPGGSLRLSCAASGFTVSSNYMSWVRQAPGKGLEWVSV**HGGST**YYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCA**RDGLGVAFDI**WGQGTMVTVSS**ER**
EVQLVETGGGLIQPGGSLRLSCAASGFTVSSNYMSWVRQAPGKGLEWVSV**HGGST**YYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCA**RAVAGPYFDY**WGQGTLVTVSS**ER**

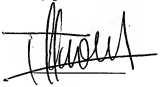
Figure 6 *Example of two sequences of rearrangements in naïve B cells from a mouse carrying the completely human immunoglobulin locus shown in Figure 4. The largest box shows the CDR3 region, the second largest box the CDR2 region, the box on the right the start of the hinge region going into constant domains and the smallest box inside the CDR2 box a somatic mutation. The sequences show that different rearrangements take place and that somatic mutations are introduced.*

Thus the genetic background used has no bearing on the expression or otherwise of heavy chain only antibody. Any non-human mammal which has a functional IgH locus can therefore be used to express IgH transgenes (devoid of CH1) the object of this invention.

6. The final point I wish to address is the contribution or otherwise of Ledbetter et al. Ledbetter et al. uses a natural endogenous camelid heavy chain only gene loci in the camel in order to generate a functional antigen specific heavy chain only antibody in response to antigen challenge. Thus, V, D, J rearrangement and subsequent affinity maturation occurs in the camel, not in a transgenic non-human mammal. Ledbetter fails to describe, much less generate, a transgenic animal capable of producing heavy

chain only antibody in response to antigen challenge, however. Ledbetter does not enable the derivation of novel heavy chain only antibodies (devoid of CH1) antibodies in the proposed non-human mammalian background as a result of antigen challenge.

7. I hereby declare that all statements made herein are of my own knowledge true and statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.



13 November 2007

Date

Dr. Frank Grosveld

Antigen-specific human monoclonal antibodies from mice engineered with human Ig heavy and light chain YACs

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We describe a strategy for producing human monoclonal antibodies in mice by introducing large segments of the human heavy and κ light chain loci contained on yeast artificial chromosomes into the mouse germline. Such mice produce a diverse repertoire of human heavy and light chains, and upon immunization with tetanus toxin have been used to derive antigen-specific, fully human monoclonal antibodies. Breeding such animals with mice engineered by gene targeting to be deficient in mouse immunoglobulin (Ig) production has led to a mouse strain in which high levels of antibodies are produced, mostly comprised of both human heavy and light chains. These strains should provide insight into the adoptive human antibody response and permit the development of fully human monoclonal antibodies with therapeutic potential.

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Fully human antibodies, with lower immunogenicity and more desirable pharmacological properties than engineered mouse antibodies, may fulfill the enormous potential for monoclonal antibodies (mAbs) in treating human disease. As the use of human B cells as a source of rearranged human antibody genes may limit the generation of therapeutically useful specificities, particularly when the target antigen is of human origin, attention has focused on the use of transgenic mice bearing unrearranged human immunoglobulin (Ig) genes to exploit the adaptive immune response of the mouse[1–4]. So far, however, the ability to generate antigen-specific human antibodies in mice has proved elusive. Mice bearing minigene constructs rearrange and express human Ig genes, but the highly skewed or aberrant, fetal-like human Ig repertoires produced in such mice, and the low expression of human Ig relative to endogenous mouse Ig[2–4], has precluded the demonstration of antigen-specific human antibodies. Hence the need for large germline segments of human Ig genes with larger variable gene repertoire and critical regulatory elements to achieve normal levels of expression and diversity is suggested.

Here we describe a novel strategy which permits the generation of mouse hybridomas making antigen-specific human mAbs and the creation of a mouse strain in which the majority of Ig produced are fully human. Using technology we have recently developed[5], yeast artificial chromosomes (YACs) carrying large segments of the human heavy and κ chain loci have been introduced into the mouse germline via fusion of yeast spheroplasts with mouse embryonic stem (ES) cells. These mice produce a broad adult-like repertoire of human Ig and are capable of giving rise to antigen-specific human mAbs upon immunization. Breeding of such mice with those whose heavy and κ genes have been inactivated by gene targeting has led to the creation of a strain which primarily produces fully human antibodies. Such mice may be exploited to elucidate the nature of the human humoral immune response upon infection or immunization, and to develop fully human therapeutic mAbs.

Human Ig YACs in ES cells and mice

YACs containing sequences from the human heavy and kappa chain loci (Fig. 1) were shown to be in intact, germline configuration (M.J.M. *et al.*, manuscript in preparation). The cloned heavy chain YAC (220 kb insert) contains the mu (μ) and delta (δ) constant (C) regions, all six functional joining (J) regions, the major diversity (D) cluster, the intronic enhancer and five most proximal variable (V) genes from four V_H families: V_{VI}, V_{V-2}, V_{I-3}, V_{IV-4} and V_{II-5} (Fig. 1)[8,9]. The cloned κ YAC (170 kb insert) contains the κ deleting element (Kde), the intronic and 3' enhancers, the $C_κ$ region, all five functional J regions and the three most proximal $V_κ$ regions in the B cluster (B1, B2, B3) (Fig. 1)[10]. A human HPRT selectable marker[11] was targeted into the right vector arm of each YAC (Fig. 1; M.J.M. *et al.*, manuscript in preparation).

HPRT-targeted human heavy (yH1) and κ (yK1) chain YACs were introduced into the HPRT-deficient ES cell line E14.TG3B1 (H.T. *et al.*, manuscript in preparation) by yeast spheroplast-ES cell fusion[5]. Seven and 11 HPRT$^+$-ES clones obtained by fusion of yH1- or yK1-containing yeast, respectively, were analysed by Southern blotting for the integrity of the YACs. Five yH1-containing clones (2B, 2C, 3A, 125A, 125E) and 10 yK1-containing clones contained all *Hin*dIII fragments detected by probes

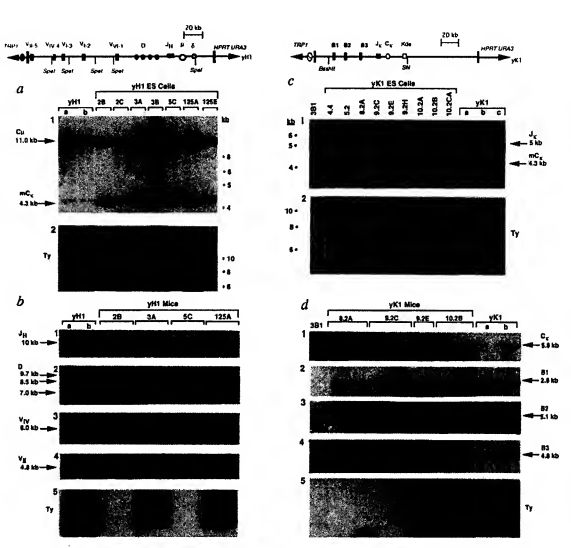

Fig. 1 Characterization of human heavy and κ light chain YACs integrated in ES cells and transgenic mice. Schematic representations of the human heavy (yH1) and kappa (yK1) YACs, retrofitted with a HPRT minigene, are shown above *a* and *c*, respectively. The locations of specific Ig regions are indicated along with YAC vector elements: ▶, telomere; ◯, centromere; ▮▮, *Eco*RI cloning site and yeast selectable markers, TRP1 and URA3. (YAC arms are not shown to scale.) *a*, *b*, Southern blot analysis of *Hin*dIII-digested DNA (10 μg) from: *a*, yH1-containing ES clones: 2B, 2C, 3A, 3B, 5C, 125A and 125E, probed with human Cμ and mouse Cκ (*a1*) and yeast Ty sequences (*a2*); *Hin*dIII-digested yH1, embedded in agarose, was used in *a*, resulting in slightly slower-migrating fragments than the corresponding fragments from yH1 in ES cells prepared in solution. *b*, mice (*2* individual offspring from each strain) generated from clones: 2B, 3A, 5C, 125A, and yH1-containing haploid yeast DNA (a= 40 ng and b=20 ng corresponding to 2 and 1 YAC DNA copies, respectively). Probes: J_H (*b1*), D (*b2*), V_H (*b3*, the band detected above the 8 kb V_H fragment in the 2B lane represents a partial digest), V_B (*b4*) and yeast Ty sequences (*b5*). *c*, *d*, Southern blot analysis of *Hin*dIII-digested DNA (10 μg) from: *c*, unmodified E14.TG3B1 (3B1) and yK1-containing ES clones: 4.4, 5.2, 8.2A, 9.2C, 9.2E, 9.2H, 10.2A, 10.2B and 10.2CA and *d*, mice (*2* individual offspring, except 9.2E) generated from clones 8.2A, 9.2C, 9.2E and 10.2B, yK1-containing haploid yeast DNA (a=10 ng, b=20 ng and c=40 ng (when shown), representing 0.5, 1 and 2 YAC DNA copies, respectively). Probes: $J_κ$ and mouse $C_κ$ (*c1*, *d1*), B1 (*d2*), B2 (*d3*), B3 (*d4*) and yeast Ty sequences (*c2*, *d5*). Fragment sizes (in kb) are indicated. *e*, Identification by PCR analysis of HuAb mice (1, 3 and 10) in a mouse litter (1–12) derived from the mating of yH1- and yK1-bearing mice. Specific human heavy chain (D, 230 bp), κ chain ($J_κ$, 860 bp) and mouse γ interferon receptor (INF-γR, 550 bp)-PCR products are indicated in control 129xC57Bl/6 mice (C), heavy (yH1)- or κ (yK1)-containing mice, or yH1;yK1 progeny.

Table 1 Structural integrity of human heavy and κ light chain YACs in ES clones and their expression in mice

a

yH1 ES Cell Clone	Cδ 7.8 kb	Cμ 11 kb	JH 10 kb	D 9.7, 8.5, 7 kb	Vγ1 ~28 kb	Vι >12, 1.7 kb	Vγ 8 kb	Vε 4.8 kb	Yeast Genomic Sequences	YAC copy number
2B	+	+	+	+	+	+	+	+		1
2C	+	+	+	+	+	+	+	+	+	2
125A	+	+	+	+	+	+	+	+	+	1
123E	+	+	+	+	+	+	+	+		1
3A	+	+	+	+	+	+	+	+	+	1
3B	+	+	+	9.7 kb 8.5 kb	+	+	+	+	+	~8
5C	+	+	+	+	+	+	+	-	-	

b

yK1 ES Cell Clone	Kde 2.5 kb	Cκ 5.8 kb	Jκ 5 kb	Bδ 4.8 kb	Bδ 5.1 kb	Bι 2.8 kb	Yeast Genomic Sequences	YAC copy number
4.4	+	+	5.5 kb	4.7 kb	4.9 kb	+	+	1
5.2	+	+	+	+	+	+	-	1
8.2A	+	+	+	+	+	+	-	1
9.2E	+	+	+	+	+	+	+	2
9.2C	+	+	+	+	+	+	-	1
10.2B	+	+	+	+	+	+	+	1
9.2H	+	+	+	+	+	+	-	~3
9.2F	+	+	+	+	ND	+	-	1
10.2C	+	+	+	+	ND	+	-	1
10.2A	+	+	+	+	+	+	+	2
10.2CA	+	+	+	+	+	+	+	2

c Human Heavy Chain

yH1 Mouse Clone	yH1 Copy Number	hμ Expression (μg/ml)
*3B	~8	3.8
125A	1	0.9
5C	1	0.8
2B	1	0.7
3A	1	0.4
2C	2	0.4
*125E	1	0.2
Control	0	0.0

d Human Kappa Chain

yK1 Mouse Clone	yK1 Copy Number	hκ Expression (μg/ml)
5.2	1	30.0
10.2B	1	27.5
9.2C	1	17.7
*9.2H	~3	15.5
*9.2F	~3	11.0
9.2E	2	10.5
*10.2C	1	8.1
8.2A	1	8.0
4.4	1	0.0
Control	0	0.0

a, b, HPRT⁻-ES clones, generated by fusion of ES cells with yH1- (*a*) or yK1- (*b*) containing yeast spheroplasts, were analysed for the presence of human heavy or κ chain-specific sequences, and yeast genomic sequences (see Methodology). The presence of the expected fragment sizes for the specific probe (as shown), and of varied levels of yeast genomic sequences are indicated as '+'. Altered-sized fragments are indicated. In the case of yH1- ES clone 3B, the 7 kb doublet from the D region was deleted. ND-not determined. *c, d*, Detection of human heavy and κ chains in mouse sera. Serum samples derived from yH1- (*c*) or yK1- (*d*) containing transgenic or chimaeric (*e*) mice, from the indicated ES clones, or non-transgenic littermate mice (control), were analysed by ELISA for hμ or hκ. Shown are representative serum titrations for individual mice.

spanning the entire respective inserts (Fig. 1, Table 1). Deletions within the D or V_H regions of yH1 were detected in clones 3B and 5C, respectively, and altered Jκ, B3 and B2 fragments of yK1 were detected in clone 4.4. All clones retained the HPRT-containing right vector arm. All yK1-containing clones and four out of seven yH1-containing clones (2C, 3A, 3B, 125A) retained an intact left arm (data not shown). All clones contained a single YAC integration except 2C and 3B (yH1) and 9.2E, 9.2H, 10.2A and 10.2CA (yK1). Hybridization with yeast repetitive probes (Ty, Y', δ, rRNA) demonstrated the presence of varying amounts of yeast genomic sequences in 4 yH1 ES clones (2C, 125A, 3A, 3B) and 5 yK1 clones (4.4, 9.2E, 10.2B, 10.2A, 10.2CA) and their absence in the remaining ES clones (Fig. 1, Table 1).

The integrity of introduced YACs was further assessed by pulsed-field gel electrophoresis following digestion with *Not*I (a site absent in yH1) or *Sfi*I (a site occurring once in yK1, generating a 105 kb fragment containing the Kde-B1 region). A single, unique fragment hybridizing to multiple yH1 or yK1 specific probes was demonstrated in 5 yH1 (2B, 3A, 5C, 125A and 125E) and in 6 yK1 (5.2, 8.2A, 9.2C, 9.2F, 10.2B, 10.2C) ES clones (data not shown; M.J.M. *et al.*, manuscript in preparation). Together, these results strongly suggest that these clones contain a single, structurally intact YAC.

YAC-containing chimaeric mice were generated from 7 yH1 and 9 yK1 ES clones. Approximately 50% of their agouti offspring contained in their germline the respective YAC and yeast sequences (when present) in unaltered form (Fig. 1).

Human Ig production in mice

The expression of the human heavy mu (hμ) and kappa (hκ) chains on B cells and in serum of yH1- and yK1-containing mice was investigated by flow cytometry and

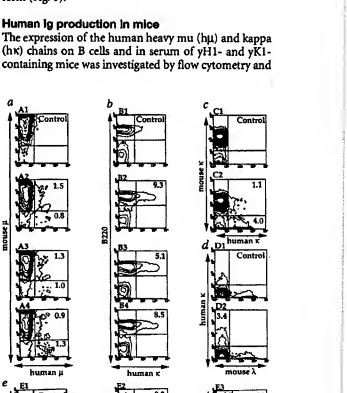

Fig. 2 Surface expression of human μ and κ chains on yH1- and yK1- containing mouse B cells. Blood or spleen lymphocytes derived from yH1-(*a*), yK1-(*b,c,d*) and yH1;yK1- (HuAb) (*e*) containing mouse strains or control mice (129xC57Bl/6:A1, B1, C1, D1, E1) were analysed by 2- or 3-colour flow cytometry for surface expression of human μ or κ chains, using antibodies to the B cell-specific marker B220 in combination with anti-human μ or κ, and anti-mouse μ, κ or λ, respectively. The net percentage of positively-stained cells (obtained by subtracting the background staining of each control) is shown in each quadrant. *a*, Blood samples from control (A1), or yH1-mice generated from clones 2B (A2), 5C (A3) and 125A (A4), were gated on B220⁺ cells and assayed for migM⁺ and migM⁻, and hμ. *b*, Spleen lymphocytes from control (B1) or yK1-containing mice: 9.2C (B2), 8.2A (B3), and 10.2B (B4) were assayed for B220 and hκ. *c, d*, Spleen lymphocytes from control (C1, D1) or yK1-containing mice 8.2A (C2, D2), gated on B220⁺ cells and assayed for expression of hκ and mκ (*c*) or gated on B220⁺ cells and assayed for expression of hκ and mλ (*d*). The percentage of mλ⁺ B cells was similar (approximately 6%) in D1 and D2. *e*, Blood samples derived from control (E1) or HuAb strains (E2: 8.2A;125A, E3: 8.2A;5C) were analysed by 3-colour flow cytometry for surface expression of human μ and κ on B220⁺ cells. The net percentage of positively-stained cells is shown in each quadrant. The FACS profiles shown are representative of five experiments performed on these strains. Similar analysis indicated the presence of hμ⁺/hκ⁺ populations in other HuAb strains: 8.2A;2B (0.27%), 9.2C; 125A (0.13%) and 9.2C;2B (0.11%).

Table 2 Repertoire analysis of human heavy chain transcripts expressed in transgenic mice

a

Clone	Frame	V	M	D	N	J
p40	in	V_H6	GCGAGACA TCG	H1	GGGTATAG	TGGGAGCCC J4
p43	out	V_H4	GCGAGAGA GGA	H1	GGTATACGACGTgGC	GGGTACA J3
p46	in	V_H6	GCAA	J1/5	TTACTATGATAGTAGTGGTTATTAC	TGAGCATTACT J4
p35	in	V_H1	GCCAGA CGG	H4	CGATTTTGTAGATGGTTA	AGAGAGCTCC J6
p55	in	V_H1	GCGAGAGA GAGGC	H1	GTATAACTGGAACTAC	GGAG J6
p56	in	V_H6	GCA	H5	GGTGTAACTGGAACT	J5
p45	in*	V_H1	GCG GTCC	XP1	GTATTACGATTTTTGACTGGTTA	CCCCTGAT J3
p73	in	V_H4	GCG C	K1	GTGGATATATGGCTACTAGAT	J3
p80	out	V_H4	GCGAGAGA	H1	ACTGGAACTAC	CGGG J6
p85	in	V_H1	GCGAG GGGGG	H1	TAGCAGtcgGCTGGTAC	CTGA J6
p87	in	V_H1	GCGAGAGAG	H4	TATAGCAGCCTCGTC	J4
p89	in	V_H1	GCGAGA AGCGCG	H1	AGCAGCAGCT	TCTC J4
p90	in	V_H1	GCGAGAG GAGGGGCCCATT	LR3	GTGGTGGTtgcTGCT	CTACGTAC J6
p97	in	V_H6	GCAAGA	LR2	GTGGgAGCT	ACCCT J4
p98	out	V_H6	GCAAGAGA TGAGGGGA	A4	TGACTACAGTAAC	CC J4
p100	in	V_H6	GCAAGAGA GGGAGCAGTGGC	J1/5	TGGTTATYAC	J6
p102	in	V_H6	GCAAGAGA	H1	TTACTGGGAC	ACGT J6
p104	in	V_H6	GCA	A1	GACTACAGTAACT	J4

Clone	Repertoire
p40	ACTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA
p43	CTTTGATATCTGGGGCCAAGGGACAATGGTCACCGTCTCTTCA
p46	ACTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA
p35	TACTACTACTACGGTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA
p55	TACTACTACTACGGTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA
p56	AACTGGTTCGACCCCTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA
p73	GCTTTTGATATCTGGGGCCAAGGGACAATGGTCACCGTCTCTTCA
p80	ACTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA
p85	TTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA
p87	CTTTGACTATCTGGGGCCAAGGGACAATCGTCACCGTCTCTTCA
p89	CTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCCCTCCTCA
p90	TACTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA
p97	CTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA
p98	GGGCCAGGGAACCCTGGTCACCGTCTCCTCA
p100	TACTACTACTACGGTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA
p102	CTACTACGGTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA
p104	TTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA

b

Clone	FR3	CDR3	FR4
p40	AR	DRGIYGARFDY	WGQGTLVTVSS
p46	A1	TMIVVITSDYYYYGMDV	WGQGTTVTVSS
p50	AR	GRFLEWLRGYYYYGMDV	WGQGTTVTVSS
p55	AR	ERRITGYFDYYYYGMDV	WGQGTTVTVSS
p56	AQ	IYGTWWFDP	WGQGTLVTVSS
p73	AR	GYRGYYGMDV	WGQGTTVTVSS
p85	AR	GSSGSWYLIDY	WGQGTLVTVSS
p87	AR	EYSSSSYYFC	WGQGTLVTVSS
p89	AR	RASSSFSFDY	WGQGTLVASSS
p90	AR	GGAHCSGGSCSYYYFDY	WGQGTLVTVSS
p97	AR	VGATLFDY	WGQGTLVTVSS
p100	AR	NGAVAGTYYYYYGMDV	WGQGTTVTVSS
p102	AR	DMNYITTTVMTV	WGQGTTVTVSS
p104	AD	YSNYDH	WGQGTLVTVSS

c

	V_{Hz}	V_{HV}	V_{λ}	$V_{H\kappa}$	Total	Human PBL(ref.12)
J_1	0	0	1	0	1 (1%)	5%
J_2	0	0	1	0	1 (1%)	0%
J_3	0	5	7	3	15 (15%)	8.1%
J_4	0	8	16	21	45 (44%)	52.2%
J_5	0	0	1	4	5%	15.2%
J_6	0	3	7	7	17 (16%)	19.2%
Total	0 (0%)	16 (15%)	33 (42%)	33 (33%)	83	(100%) 100%

Human μ-specific mRNAs were amplified by PCR, cloned and analysed by sequencing or by hybridization to V_H- and J_H-region specific probes. *a*, Nucleotide sequences of 18 unique human heavy chain clones are divided into V_H, D, J_H and N segments, as identified by homology with published germline sequences[6,7,28–31]. Whether each V–D–J junction is in or out of open reading frame is indicated. Each D segment assignment is based on at least 8 bases of homology. Differences from the published sequences are in lower case. N-segment nucleotides were determined by their lack of sequence homology to V_H, D or J_H sequences. Clones chosen for sequencing had previously been shown to possess a V_H segment by colony hybridization (see part *c*). *b*, Predicted amino acid sequences of the 14 in-frame V–D–J junctions are divided into Framework Region 3 (FR3), CDR3 and FR4[14]. *c*, Results of colony hybridizations showing V_H and J_H gene utilization, as compared to that detected in human peripheral B cells[12]. Results are given only for colonies hybridizing to V_H, J_H and Cμ probes.

ELISA. Hμ was expressed on the surface of 1.5–2.5% of the B220⁺ cells in blood (Fig. 2*a*) or spleen (not shown) in all 5 yH1 strains analysed (2B, 2C, 3A, 5C, 125A). Approximately half of the hμ⁺ population had no detectable surface mouse mu (mμ) (Fig. 2*a*), indicating that hμ production can substantially exclude the expression of mμ. Hμ was detected in sera derived from all yH1 strains at 0.2–3.8 μg ml⁻¹ (Table 1*c*). In all 3 yK1 strains examined (8.2A, 9.2C, 10.2B), 5–9% of the B220⁺ splenocytes expressed surface hκ (Fig. 2*b*). In >80% of this population, hκ excluded the expression of mouse lambda (mλ) or kappa (mκ) (Fig. 2*c*,*d*). All yK1 mice derived from structurally intact YACs expressed hκ in serum at 8–30 μg ml⁻¹ (Table 1*d*).

Human Ig-producing (HuAb) mice containing one copy each of yH1 and yK1 were generated (Fig. 1*e*). Three-colour flow cytometry of peripheral blood lymphocytes, derived from five HuAb strains (8.2A;125A, 8.2A;5C, 8.2A;2B, 9.2C;125A, 9.2C;2B) revealed B220⁺ populations containing 0.8–3.9% hμ⁺ or 1.4–4.8% hκ⁺ cells (Fig. 2*e* and data not shown). Significantly, all five HuAb strains contained a population of B220⁺ cells (0.06–0.27%) which simultaneously expressed both hμ and hκ (Fig. 2*e* and legend). The production of serum antibodies in HuAb mice containing both hμ and hκ (hμ/hκ) was demonstrated by an ELISA in which anti-hμ antibody was used to capture and anti-hκ antibody used to detect the product. Hμ/hκ antibodies were detected in HuAb strains 8.2A;2B and 8.2A;5C at 0.7 and 0.3 μg ml⁻¹, respectively (data not shown). Thus, in HuAb mice, human heavy and kappa genes on YACs were productively rearranged and expressed, leading to the presence of a significant population of B cells expressing both surface hμ and hκ and secreting antibodies containing both human heavy and light chains in the mouse serum.

Diverse adult-like human Ig repertoire in mice

To determine the diversity of the hμ and hκ repertoire in YAC-containing mice, hμ and hκ cDNAs were cloned from mouse spleen RNA. Hybridization analysis of hμ and hκ cDNA clones revealed broad usage of the V and J genes contained in the YACs (Tables 2*c*, 3*c*). All six J_H and five J_κ segments were represented with a frequency comparable to that detected in adult human B cells[12,13]. Three of the four V_H families in yH1 were represented, with V_{H6} used about half as often as V_{H3} and V_{H1}. No V_H transcripts (Table 2) or V_H–J_H rearrangement products (data not shown) were detected, suggesting that the proximity of V_H to the YAC vector cloning site may account for its inability to rearrange. All three V_κ genes were represented, with B3 more frequently used than B2 or B1 (Table 3).

To analyse further the human Ig repertoire expressed in these mice, individual cDNA clones were sequenced. The 18 hμ cDNAs analysed displayed a V_H and J_H usage similar to that detected by hybridization (Table 2). Ten different D regions were represented, all with significant homology to known germline D genes. Some D sequences with less than 85% identity (for example, N1 in clones μ43 and μ85, and LR2 in clones μ90 and μ97) may represent novel D segments. For example, the C to T and A to G changes observed at the same position in the D segments of μ43 and μ85 (and ref. 12), suggest the existence of a new DN family member. Multiple reading frames were used in some of the D regions (μ90/μ97, μ55/μ102, μ46/μ100), suggesting a D usage more human-like than mouse[12,14]. Non-germline nucleotides (N addition) were observed in 15 (83%) of the hμ cDNAs, with a majority having N additions at both the VD and DJ junctions. The length of N addition varied between 1–12 bp (average 6.1). The majority of the in-frame clones contained a complementarity determining region 3 (CDR3) of 10–18 amino acids (average 12). The $V_κ$ and $J_κ$ usage observed for 21 sequenced hκ cDNAs also agreed with hybridization analysis. In seven of the clones, N additions of 1–4 bp were found at the V–J junction. CDR3 sequences for in-frame κ transcripts were 9–10 amino acids in length. The $J_κ$ usage and CDR3 length observed are consistent with previous results for human B cells[13].

The pattern of V_H, D and J_H usage observed in human Ig YAC-containing mice is reminiscent of adult human B cells[12,13], in contrast to human Ig minigene-bearing mice[2–4]. There is an absence of position-biased V_H and D usage, in particular, for the V_{H1} and DQ52 segments which are characteristic of human fetal development[15,16], with the latter dominating the in-frame repertoire observed in minigene-bearing mice[3,4]. Furthermore, the average length of N addition (6.1 bp), and thus the CDR3 region, closely approximates that seen in adult human B cells (7.7 bp)[12], while in minigene-bearing mice the average length (2.9 bp)[3,4] resembles that seen in adult mouse B cells (3.0 bp)[17]. These results suggest that the human Ig YACs contain sequences required to direct human-like repertoires in mice. Therefore, introduction of YACs

Table 3 Repertoire analysis of human κ transcripts expressed in transgenic mice

a

Clone	Frame	V	N	J	
K1	in	B3	AGTACTCCTC	J1	GGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGA
K2	out	B3	AGTACTCCT	TTCT	J2	GTGCAGTTTTGGCCAGGGGACCAAGCTGGAGATCAAACGA
K3	in	B3	AGTACTCC		J1	GTGGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGA
K5	out	B3	TTTTCCTC		J3	ACTTTCGGCCCTGGGACCAAAGTGGATATCAAACGA
K7	out	B3	AGTACTCCTCT		J1	GGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGA
K8	in	B3	AGTACTCCTC		J2	GCAGTTTTGGCCAGGGGACCAAGCTGGAGATCAAACGA
K9	in	B3	AGTACTT	T	J2	GTGCAGTTTTGGCCAGGGGACCAAGCTGGAGATCAAACGA
K10	in	B3	AGTACTCC		J1	GTGGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGA
K13	out	B3	AGTACTCCTC	G	J1	GGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGA
K14	in	B3	AGTACTCC	CAT	J2	GTGCAGTTTTGGCCAGGGGACCAAGCTGGAGATCAAACGA
K15	out	B3	AGTACTCC	AT	J2	GTGCAGTTTTGGCCAGGGGACCAAGCTGGAGATCAAACGA
K16	out	B2	TTCCCTC		J3	ATTCACTTTCGGCCCTGGGACCAAAGTGGATATCAACGA
K18	in	B3	AGTACTCC		J4	GCTCACTTTCGGCGGAGGGACCAAGGTGGAGATCAAACGA
K20	in	B2	TTCCCT		J1	TGGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGA
K22	out	B2	TTCCCTC		J4	ACTTTCGGCGGAGGGACCAAGGTGGAGATCAAACGA
K25	in	B3	AGTACTCC		J3	ATTCACTTTCGGCCCTGGGACCAAAGTGGATATCAACGA
K27	in	B3	AGTACTCC		J4	GCTCACTTTCGGCGGAGGGACCAAGGTGGAGATCAAACGA
K28	out	B3	AGTACT	GTC	J3	TCACTTTCGGCCCTGGGACCAAAGTGGATATCAACGA
K29	in*	B3	AGTACTCC	CAT	J2	GTGCAGTTTTGGCCAGGGGACCTAGCTGGAGATCAAACGA
K30	in	B1	TTTTCTC		J1	GGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGA
K31	out	B3	AGTACTCCTCC		J4	ACTTTCGGCGGAGGGACCAAGGTGGAGATCAAACGA
K32	in	B3	AGTACTCC		J3	ATTCACTTTCGGCCCTGGGACCAAAGTGGATATCAACGA
K33	out	B3	AGTACTCCTCC		J1	GGACGTTCGGCCAAGGGACCAAGGTGGAAATCAACGA
K34	in	B3	AGTACTCCTCC		J1	GACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGA

b

Clone	FR3	CDR3	FR4
K1	YYC	QQYYSTPWT	FGQGTKVEIKR
K3	YYC	QQYYSTPWT	FGQGTKVEIKR
K8	YYC	QQYYSTPKS	FGQGTKLEIKR
K9	YYC	QQYYSTLCS	FGQGTKLEIKR
K10	YYC	QQYYSTPWT	FGQGTKVEIKR
K14	YYC	QQYYSTPMCS	FGQGTKLEIKR
K18	YYC	QQYYSTPLT	FGQGTKVEIKR
K20	YYF	CLQHDRFPWT	FGQGTKVEIKR
K25	YYC	QQYYSTPPT	FGPGTKVDIKR
K27	YYC	QQYYSTPLT	FGQGTKVEIKR
K30	YYC	LQSKNFPRT	FGQGTKVEIKR
K32	YYC	QQYYSTPPT	FGPGIKVDIKR
K34	YYC	QQYYSTPPT	FGQGTKVEIKR

c

	B1	B2	B3	Total
$J_κ1$	4	13	108	125 (47%)
$J_κ2$	10	5	53	68 (26%)
$J_κ3$	0	2	29	31 (12%)
$J_κ4$	1	9	25	35 (13%)
$J_κ5$	0	0	4	4 (1%)
Total	15 (6%)	29 (11%)	219(83%)	263 (100%)

mRNAs containing $hC_κ$ were amplified by PCR, cloned and analysed by sequencing or by colony hybridization to $V_κ$- and $J_κ$-region specific probes. *a*, Nucleotide sequences of V–J junctions of 21 independent human κ clones are shown, divided into $V_κ$, $J_κ$ and N segments and identified based on homology to published germline B1, B2 and B3, and $J_κ$ sequences[26,28]. Also indicated is whether each V–J junction is in or out of an open reading frame. N-segment nucleotides were determined by their lack of sequence homology to neither $V_κ$ nor $J_κ$ sequences. Differences from the published sequences are in lower case. The sequences shown are those which contained a V–J joining. The one sequenced B1-containing clone lacked a translational initiation site, as described[25]. *b*, Predicted amino acid sequences of in-frame V–J junctions are divided into FR3, CDR3 and FR4[14]. *c*, Results of colony hybridizations showing $V_κ$ and $J_κ$ gene utilization. Results are given only for colonies hybridizing to $V_κ$ and $J_κ$ probes.

with larger numbers of variable genes should ultimately recapitulate the diversity seen in humans.

Ag-specific fully human mAbs from mice

To determine whether HuAb mice can mount a specific human antibody response, mice were immunized with tetanus toxin C fragment (tet C). After immunization, tet C-specific hμ and hκ were readily detected in serum (Fig. 3*a*). The human origin of the tet C-specific antibodies was confirmed by using an ELISA in which tet C was used to capture and anti-hμ or anti-hκ used to detect the bound species. Thus, upon immunization, the HuAb mice are capable of producing antigen-specific human antibodies.

To determine whether antibodies containing both human heavy and light chains were produced, splenocytes derived from tet C-immunized HuAb mice (8.2A;5C) were fused with P3X63-Ag8.653 myeloma cells, and the resulting hybridomas screened for the production of tet C-specific fully human antibodies. Analysis of 678 hybridoma culture supernatants revealed 92 hκ⁺ clones and 16 hμ⁺ clones. Three clones were found to produce fully human mAbs specific for tet C. To confirm that all of the desired properties reside within the same antibody molecule, ELISAs were used in which either tet C was used to capture and anti-hκ used to detect the bound species (Fig. 3*b*), or anti-hμ used to capture and anti-hκ used to detect the bound species (Fig. 3*c*). All three clones were positive in both assays, indicating that HuAb mice, although containing only a small fraction of B cells co-expressing hμ and hκ, can mount an antigen-specific response leading to the generation of fully human mAbs. The nature of the human repertoire associated with the tet C-specific response and the extent to which somatic mutation plays a role in the maturation of tet C-specific human antibodies is currently under investigation.

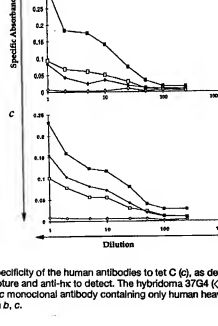

Fig. 3 Production of tet C-specific human polyclonal (●) or monoclonal (*b*, *c*) antibodies by HuAb mice. *a*, Sera obtained from HuAb strain 8.2A;125A, non-immune (○, ◆) or hyperimmune to tet C (□, ■) were analysed by ELISA for tet C-specific hμ (□) and hκ (■) antibodies. Similar results were obtained from HuAB strains 8.2A;2B, 8.2A;5C and 9.2C;2B. *b*,*c*, Supernatants derived from hybridomas (42C7-■,45B5-□, 45D9-◆, control 37G4-○), generated by fusion of hyperimmune HuAb strain 8.2A;125A splenocytes with mouse myeloma cells, were analysed for the presence of fully (hμ/hκ) human monoclonal antibodies (*b*) and for specificity of the human antibodies to tet C (*c*), as detected in ELISA using tet C to capture and anti-hκ to detect. The hybridoma 37G4 (○), secreting a tet C-specific monoclonal antibody containing only human heavy chain was used as a control in *b*, *c*.

Human YACs restore B cell and Ig production

While HuAb mice can mount an antigen-specific human antibody response, the preferential expression of mouse Ig genes suggested the potential value of their inactivation to increase the production of fully human antibodies in mice. Therefore, yH1- and yK1-expressing mice were bred with mice engineered by gene targeting to be deficient in mouse Ig production.

Initially, we examined the ability of yH1 to induce proper mouse B cell development and production of human Ig in a strain containing two functionally inactivated mouse heavy chain alleles (yH1;$\Delta J_H/\Delta J_H$). $\Delta J_H/\Delta J_H$ parental mice fail to rearrange their heavy chain genes, leading to a complete absence of mature B cells and a complete block in Ig production[18]. In yH1;$\Delta J_H/\Delta J_H$ mice, reconstitution of mature B cells (B220⁺,hμ⁺) was observed in bone marrow (Fig. 4*a*), spleen and blood (Fig. 4*e*) corresponding to 58%, 55% and 30% of the levels in wild-type mice, respectively. The majority of B220⁺/hμ⁺ cells in blood also expressed human delta (hδ) and all of the hδ⁺ cells co-expressed hμ (data not shown), indicating proper expression and regulation of the human constant regions in these mice[19]. Remarkably, yH1;$\Delta J_H/\Delta J_H$ mice produced serum hμ at 350 μg ml⁻¹, a level 510-fold greater than parental 125A mice (Table 1*c*) and within 2–3-fold of normal human IgM serum levels. Hμ serum titres showed an age-dependent increase (not shown). These results demonstrate that yH1 can reconstitute B cell development in mice deficient in mouse heavy chains and direct the expression and assembly of high levels of hμ/mouse light chain antibodies.

The yH1;$\Delta J_H/\Delta J_H$ mice were further evaluated by analysing the orderly differentiation of bone marrow-derived B cells by flow cytometry using antibodies to the cell surface marker CD43, which defines early B cell subpopulations[20], in conjunction with antibodies to B220 and hμ. Bone marrow from $\Delta J_H/\Delta J_H$ mice contained no mature B cells ($B220^{high/intdull}$, $CD43^-$; R1, R2 populations) and a population (5.5%) of pro-B cells ($B220^{dull}$, $CD43^+$; R3 population), comparable to wild-type mice (Fig. 4*a*). In contrast, yH1;$\Delta J_H/\Delta J_H$ bone marrow contained a nearly normal R1, R2 population (41%) of mature B cells; however, the size of the pro-B cell population (12.4%) was increased (Fig. 4*a*). Surface hμ was detected only in the B220⁺, $CD43^-$ population of yH1;$\Delta J_H/\Delta J_H$ mice, similar to mμ in wild-type mice (Fig. 4*b*). Thus, while B cell development and Ig production are substantially restored in yH1;$\Delta J_H/\Delta J_H$ mice, the maturation of bone marrow-derived B cells may be somewhat less efficient than in normal mice.

To delineate this partial block in B cell maturation, we examined the surface expression of HSA and BP-1 in bone marrow to resolve the pro-B and pre-B cell populations[20]. Large, primarily $CD43^-$ populations of B220⁺, HSA⁺ cells and B220⁺, $BP\text{-}1^-$ cells as well as a smaller $CD43^-$, B220⁺, $BP\text{-}1^+$ cell population confirmed that B cell development is largely normal in these mice (Fig. 4*c*,*d*). However,

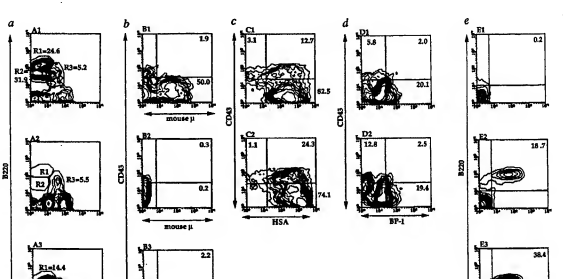

Fig. 4 Reconstitution of B cell development in ΔJ_H homozygous mutant mice by yH1 (125A strain) YAC. *a–d*, Bone marrow lymphocytes from normal control (129×B6: A1, B1, C1, D1), $\Delta J_H/\Delta J_H$ (A2, E1) or yH1:$\Delta J_H/\Delta J_H$ mice (A3, B2, B3, C2, D2) were assayed for surface expression of CD43 and B220 (*a*), or gated on B220$^+$ cells and assayed for surface expression of mμ and CD43 (B1, B2), hμ and CD43 (B3), HSA and CD43 (*c*), or BP-1 and CD43 (*d*). The bone marrow B220$^+$ sub-populations (R1, R2 and R3 (high (bright) or low (dull) levels of surface B220$^+$ are delineated)) are indicated with their respective percentages of positively stained cells. *e*, Peripheral blood (E1, E2) and spleen (*E3*) lymphocytes were assayed for hm and B220 in $\Delta J_H/\Delta J_H$ (E1) and in yH1:$\Delta J_H/\Delta J_H$ mice (E2, E3). The net percentage of positively-stained cells is shown in each quadrant. In the normal 129×B6 mouse, 64% of the cells in blood and spleen were B220$^+$. All animals used were 3 month old males.

increased populations of CD43$^+$, HSA$^+$ cells and CD43$^+$, BP-1$^-$ cells as well as smaller populations of CD43$^+$, HSA$^-$ cells and brighter CD43$^+$, HSA$^+$ cell populations indicated a small accumulation of pro-B cells, suggesting a less efficient pro-B cell to pre-B cell transition, the stage at which V to DJ joining occurs[28].

We next evaluated the ability of yH1 and yK1 together to restore B cell development and Ig production in a strain called Xenomouse, which also contains two functionally inactivated mouse heavy and kappa light chain alleles (yK1:yH1:$\Delta J_H/\Delta J_H$:$\Delta C_\gamma/\Delta C_\gamma$). In the $\Delta J_H/\Delta J_H$:$\Delta C_\gamma/\Delta C_\kappa$ parental mouse, called DI (for double-inactivated), the expression of both mouse heavy and κ was blocked (manuscript in preparation). In contrast to DI mice which did not produce any mature B220$^+$ cells (Fig. 5*a1–c1*), mature B220$^+$ cells were present in Xenomouse at 10% of the level seen in the wild-type. Approximately half of these cells (43%) co-expressed hμ and hκ, while the remainder (57%) co-expressed hμ and mλ (Fig. 5*a2*, *b2*). No co-expression of mλ and hκ was detected, indicating that each light chain completely excluded the expression of the other (Fig. 5*c2*). Higher levels of fully human hμ/hκ antibodies (10 μg ml^{-1}) than hμ/mλ antibodies (3 μg ml^{-1}) were detected in Xenomouse. The level of hμ/hκ was at least several hundredfold higher than the level detected in the parental HuAb strain (9.2C;2B), confirming that inactivation of the mouse heavy and κ genes greatly increased levels of fully human antibodies. In addition, the relative level of B cells expressing only hμ and hκ in Xenomouse was 100–200-fold higher than in the HuAb strains from which antigen-specific human antibodies were obtained, suggesting the usefulness of Xenomouse in deriving fully human mAbs. Higher levels of hμ/hκ antibodies (200 μg ml^{-1}) were detected in another Xenomouse strain (8.2A; 2B; $\Delta J_H/\Delta J_H$:$\Delta C_\gamma/\Delta C_\kappa$). As HuAb strains producing higher levels of hμ/hκ are bred with DI mice to create additional Xenomouse strains, it is anticipated that higher levels of B cell reconstitution and antibody production will be attained.

Discussion

We have produced antigen-specific, fully human mAbs in mice and created mouse strains in which the majority of antibodies produced are fully human. The ability to derive antigen-specific human antibodies upon immunization of mice may be related to the diverse human adult-like repertoire observed following the introduction of large, intact germline segments of the human heavy and κ loci contained on YACs, in contrast to the abnormal repertoires seen in human Ig minigene-bearing mice[1–4]. While the human Ig repertoire of our YAC-containing mice are characterized by the V, D and J usage, length of N addition and CDR3 size observed for adult human B cells[29,13], earlier studies with minigenes revealed fetal-like, position-biased usage of D segments and abnormally small N addition and CDR3 size[3,4], and abnormal CDR3 sequences[2]. These differences, as well as the higher, less position-dependent expression levels noted for YAC-containing mice, may reflect the greater size, variable gene content, structural integrity upon integration, and/or presence of unidentified regulatory elements needed for optimal expression and proper regulation.

The possibility that human Ig genes compete inefficiently with mouse antibody genes by virtue of intrinsic activity or fewer V segments, led us to investigate human Ig expression in mice with inactivated mouse Ig genes. Together, human heavy and κ YACs restored B cell development in mice with inactivated mouse heavy and κ genes, and the majority of antibodies produced are fully human. Indeed, as half of the mature B cells of these mice

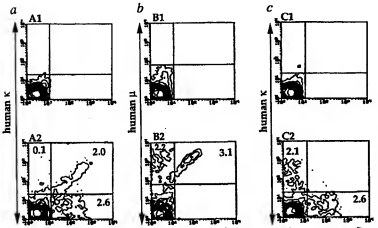

Fig. 5 Reconstitution of B cell development in Xenomouse. Peripheral blood mononuclear cells derived from a 3.5 week old DI ($\Delta J_H/\Delta J_H$; $\Delta C_\mu/\Delta C_\mu$) mouse (A1, B1, C1) or Xenomouse (9.2C; 2B; $\Delta J_H/\Delta J_H$; $\Delta C_\mu/\Delta C_\mu$) (A2, B2, C2) were assayed for hμ and hκ (a), hμ and mλ (b) or hκ and mλ (c) as described in Methodology. The net percentage of positively stained cells is shown in each quadrant. All hμ+, hκ+ and hμ+, mλ+ cells were B220+. In the normal mouse, 47% of peripheral blood mononuclear cells were B220+. DI mice were generated by the breeding of homozygous ΔJ_H-mutant ($\Delta J_H/\Delta J_H$) mice[18] and homozygous ΔC_μ-mutant ($\Delta C_\mu/\Delta C_\mu$) mice. $\Delta C_\mu/\Delta C_\mu$ mice were derived by gene-targeted deletion of the mouse C_μ region (manuscript in preparation.)

express surface hμ and hκ, but not mλ, and the relative level of hμ/hκ B cells is at least 100-fold higher than in HuAb strains used to derive antigen-specific human antibodies, such mice should be extremely useful for obtaining human monoclonal antibodies upon immunization. The yH1 YAC induced the maturation of the growth-arrested B cell lineage in homozygous ΔJ_H-mutant mice, leading to the production of hμ levels approaching those in normal human serum. These results suggest that hμ can readily assemble with mouse B cell receptors, allowing the efficient development of mature, functional B cells. The analysis of bone marrow-derived B cells suggests that in yH1;$\Delta J_H/\Delta J_H$ mice the progression of late pro-B to pre-B cells is somewhat less efficient than in normal mice, a point at which V-DJ rearrangement is initiated[20]. The limited number of V genes in yH1 may result in a lower frequency of rearrangement and thus incomplete B cell development (analysis in progress), suggesting the value of a greater number of human V genes to support more complete B cell maturation and the generation of even more complex repertoires.

The ability to produce a diverse repertoire of fully human monoclonal antibodies may have significant application to human therapy. Unlike humanized mouse antibodies which contain a significant number of residues from murine hypervariable regions, fully human antibodies may be less immunogenic, and thus more suited for repeated administration, as they would present only minor idiotypic variations from any given patient. Such mice lack immunological tolerance to and thus readily yield antibodies to human proteins, which may constitute an important class of therapeutic targets. The introduction of larger portions of the human heavy and light chain loci may ultimately yield strains of mice capable of recapitulating the full repertoire characteristic of the human humoral response to infection or immunization. This strategy of introducing large segments of the human genome into mice coupled with inactivation of the corresponding mouse genes may also have applicability to the investigation of other complex or uncharacterized loci.

Note added in proof: Hybridomas producing human antibodies against a human protein, IgE antibody, have been generated.

Methodology

Generation and DNA analysis of yH1- and yK1-containing ES cells and mice. yH1 (240 kb) and yK1 (195 kb) were identified from the Washington U. human-YAC library DNA using V_H PCR primers and a C_μ probe, respectively (M.J.M. *et al.*, manuscript in preparation). yH1- and yK1-containing yeast were fused with E14.TG3B1 cells as described[2] and HAT-resistant colonies were expanded for analysis. Probes used: D (detecting three expected *HindIII* bands, 9.7 kb, 8.5 kb and 7.0 kb (as a doublet), of D1–D4 family segments), C_μ, C_δ, J_H, V_{HB} V_J and V_{HC} as described in ref. 21; $V_H{}^{23}$, C_κ (ATCC, 59173), $J_\kappa{}^{2,3,4}$, $B1^{24}$, $B2^{25}$, $B3^{26}$, $mC_\gamma{}^{27}$ and yeast Ty^2. The number of integrated YACs per ES cell was determined by densitometric measurements of human Ig hybridization signals, normalized to those of mouse C_μ probe, and compared to the signal intensity of YACs in yeast. An equivalent hybridization signal should be obtained from a single-copy sequence in 20 ng of haploid yeast DNA and in 10 μg of diploid mammalian DNA.

Chimaeric mice were generated by microinjection of ES cells into C57Bl/6 blastocysts. HuAb offspring were identified by PCR analysis of tail DNA (30 cycles of 1 min at 94 °C; 2 min at 60 °C; 3 min at 72 °C) using human-specific primers for D: (DXPA: 5'–GCAATTACTAGTATAGACCCGAGTGTCCCC–3'; and DXPB: 5'–GCAATGGTCGACGGTTTTTGATGGACTCTG–3'; 230 bp fragment), and J_H: (J_H0: 5'–CTCGAAAAGGGAGTTGAGCT TCAGCAGCTG–3'; and J_H3: 5'–GATACAATGGCACTAAAATC TCACG–3'; 860 bp fragment) and control primers for the mouse γ interferon receptor (IFN-γR) (5'–TTGGATTCTGGTGGTTGCTC–3' and 5'–GACCTATTTGTGCATTGGAAGC–3'; 550 bp fragment). The size markers (M) are a 1 kb DNA ladder (Gibco/BRL).

Flow cytometry analysis. Peripheral blood, spleen and bone marrow lymphocytes obtained from 4–12 week old transgenic or control mice were purified on Lympholyte M (Accurate) and treated with purified Fc γ II receptor (Pharmingen, 0124 1D) to block non-specific binding to Fc receptors, stained with antibodies and analysed on a FACScan (Becton Dickinson, LYSIS II software). Antibodies used: CyChrome anti-B220 (Pharmingen, 01124A); fluorescein isothiocyanate (FITC) anti-hIgM (Pharmingen, 08074D); phycoerythrin (PE) anti-mIgM^a (Pharmingen, 05095B) and PE anti-mIgM^b (Pharmingen, 05105B); biotin anti-hδ (Southern Biotechnology Association, 9030-08); biotin anti-hκ (Pharmingen, 08172D); FITC anti-mλ (Pharmingen, 02174D); FITC anti-CD43 (Pharmingen, 01604D); PE anti-CD43 (Pharmingen, 01605B); biotin anti-6C3/BP-1 (Pharmingen, 01282D); PE anti-HSA (Pharmingen, 01575A). PE-streptavidin (Pharmingen, 13025D) was used to detect biotinylated antibodies.

To assay hκ versus mκ expression in yK1 transgenic mice, mouse peripheral blood lymphocytes were first incubated with rat IgG (Pierce, 31233) then stained with PE-conjugated goat anti-mκ (Southern Biotechnology Association, 1050-09), washed, incubated with mouse IgG (Pierce, 31204), then stained with FITC-conjugated mouse anti-hκ (Pharmingen, 08174D) and CyChrome-conjugated anti-B220.

ELISA assays. Sera were obtained from 4–12 wk old transgenic or chimaeric (containing 40–90% of ES cell-derived B cells) mice. Human serum μ was assayed using mouse monoclonal anti-hμ (AMAC, Clone A6) immobilized on Nunc Immunoplates (Maxisorp F96) and detected with biotinylated goat anti-hμ (Caltag, preabsorbed with normal mouse serum to lower background due to cross reactivity). Similarly, hκ was assayed using goat anti-hκ (Vector) to capture and detected with biotinylated goat anti-hκ (Vector). The standard used to determine hμ concentrations was hIgM (Sigma, 1-

8260) shown to be equivalent to a chimaeric hIgM/mλ antibody (Serotec, MCA 446). The standard used to determine hκ concentrations was hIgG$_1$/hκ (Sigma, I-3889). Hμ/hκ antibodies were detected in serum by ELISA using mouse monoclonal anti-hμ (AMAC, Clone Af6) to capture and detected with biotinylated goat anti-hκ (Vector) using hIgM (Sigma, I-8260) as a standard. Tet C-specific antibodies (polyclonal or monoclonal) were assayed by coating plates (see above) with 100 ng tet C (Boehringer Mannheim, 1348655)/well, incubating with serial dilutions of the appropriate serum and then detecting with either biotinylated goat anti-hμ (Caltag, see above) or biotinylated goat anti-hκ (Vector). Human monoclonal antibodies were assayed the same as hμ/hκ antibodies above. Biotinylated antibodies were detected using ABC-HRP (Vector, PK-4000). Absorbance at A490 was measured using a UVmax spectrophotometer.

Immunization of mice and generation of hybridomas. Mice were immunized 4 times at about 2 wk intervals with tet C (Boehringer Mannheim 1348655) 50 μg/injection, in Freund's Complete Adjuvant (primary injection) or Freund's Incomplete Adjuvent (subsequent boosts) subcutaneously. The mice were bled 4 days after final boost and titered for human anti-tet C antibodies by plate ELISA. Serially diluted samples were incubated with 100 ng tet C bound/well of Nunc Immunoplate. The human antibody chains were then detected using the biotinylated anti-hμ and hκ antibodies (see "ELISA assays"). To generate mouse hybridomas, splenocytes from immunized mice were fused with nonproducer P3X63-Ag8.653 myeloma cells, 4–5 days following the final boost, using 50% PEG 4000 (Boehringer Mannheim). After 10–14 days the supernatants from hybridomas growing in HAT-selected medium were screened for the presence of fully human antibodies and for tet C specificity as described above.

Repertoire of human Ig transcripts expressed in transgenic mice. poly(A)$^+$ mRNA was isolated from a yH1 spleen (strain 2B) or a yK1 spleen (strain 8.2A) transgenic mouse using a FastTrack kit (Invitrogen). Human μ-specific mRNAs amplified using a 5′ Amplifinder RACE kit (Clontech), using oligonucleotide hμP1 (5′–TTTTCTTTGTTGCCGTTGGGGTGC–3′) for reverse transcription and hμP2 (5′–GGGAAGCCCCGGGTGCTGCTGATG–3′) for amplification. Human κ-specific cDNAs were amplified using hKP1 (5′-CTCTGTGACACTCTCCTGGGAGTTT-3′) for reverse transcription and hKP2 (5′–ACCCGATTGGAGGGCGTT-ATCCAC–3′) for amplification. PCR products were cloned into pCRII using a TA Cloning kit (Invitrogen) and their sequences determined by double-stranded dideoxynucleotide sequencing. For hybridization experiments, individual colonies were picked onto multiple gridded LB-amp plates and grown at 37 °C. Colonies were transferred to Genescreen (DuPont) and lysed *in situ*[28] to yield replicas of the ordered grid. DNAs immobilized on filters were probed with ^{32}P-labelled V$_H$ or Cμ fragments for yH1 cDNAs or with V$_\kappa$ fragments for yK1 cDNAs (see above). ^{32}P-end labelled J$_H$- or J$_\kappa$-specific oligonucleotides were hybridized to DNAs on filters in 1% bovine serum albumin, 1.25 mM EDTA, 0.5 M NaPO$_4$ buffer, pH 7.2, and 7.1% SDS at 38 °C except J$_H$6 which was hybridized at 30 °C. Filters were washed three times in 6× SSC for 3 min at room temperature then washed once in 6× SSC for 3 min at 34 °C with the exceptions that J$_H$6-probed filters were washed at 30 °C and J$_H$5-probed filters were washed at 42 °C. J$_H$-specific oligonucleotides were: J$_H$1:5′–ACTTCCAGCACTGGG–3′; J$_H$2: 5′–ACTTGGATCTCTGG–3′; J$_H$3: 5′–TTTTGATATCTGGGG–3′; J$_H$4: 5′–TTGGACTACT–GGGGC–3′ J$_H$5: 5′–TTCGACCCCTGGGGC–3′ J$_H$6: 5′–TACTA–CTACTACTAC–3′. J$_\kappa$-specific oligonucleotides were: J$_\kappa$1: 5′–AGGTGGAAATCAAAC–3′ J$_\kappa$2: 5′–TTTGGCCAGGGGACC–3′ J$_\kappa$3: 5′–TTCGGCCCTGGGACC–3′ J$_\kappa$4: 5′–TTTCGGCGGAGGGAC–3′ J$_\kappa$5: 5′–AGGGACAGCACTGGA–3′.

Acknowledgements

We thank R. Kucherlapati, S. Sherwin, G. Otten, K. Rajewsky, R. Germain, R. Tepper, Y. Nishi, T. Chuman, J. Hyde, K. Lenting, G. Lee, R. Ludovico, D. Villanueva, D. Casertini-Borsa and V. Lubeck for their help and contribution to this work. This work was supported in part by Xenotech, Inc., the joint venture between Cell Genesys, Inc. and JT Immunotech.

Received 20 December; accepted 17 March 1994.

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The human immunoglobulin loci introduced into mice: V (D) and J gene segment usage similar to that of adult humans

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Variable gene segments of the human immunoglobulin loci are represented in the human peripheral repertoire at different frequencies. XenoMouse™ strains contain approximately 2 megabases of the human immunoglobulin heavy and kappa light chain loci that functionally recapitulate the human humoral immune system. Analysis of human antibody transcripts from XenoMouse spleens and lymph nodes revealed that V, D and J gene segment utilization from these unimmunized animals were nearly identical to the gene segment utilization reported for humans with extensive antigenic histories.

Received	31/8/99
Revised	2/11/99
Accepted	12/11/99

Key words: Repertoire / Heavy chain / Kappa chain / XenoMouse

1 Introduction

The utilization of Ig gene segments comprising the human humoral repertoire is not random [1–6]. Specific gene segments are over-represented while other gene segments are significantly under-represented. In general, the distribution of H chain variable gene segment families reflects their germ line complexity, *i.* e. V_H3, the largest family, is found the most often, followed by V_H4 and V_H1 [4, 5, 7, 8]. However, not all the V_H gene segments within a family are represented equally [6, 9, 10]. In fact, relatively few gene segments from a V_H gene family may constitute the majority of the observed repertoire for that family. Non-random utilization of V_H gene segments has been confirmed in multiple studies employing a variety of molecular approaches, each with inherent biases, and representing repertoires of different individuals, each with unique genetic polymorphisms and distinct antigenic histories.

While initial studies focused on characterizing the gene segment utilization of the human antibody repertoire, recent studies have focused on the mechanisms of selection that shape the development of the human Ig repertoire. Antigenic history, in the context of both positive and negative selection, has been cited as an important factor in the generation of mature B cells [11–13]. B cells from different organs or lineages (fetal, intestinal, B1, B2) may contain distinct repertoires that serve specialized functions [14–17]. Analyses performed on pre-B cells suggest that development in the bone marrow and pairing with the surrogate L chain shapes the primary repertoire [18–21]. How much of the observed human antibody repertoire, including variable gene segment bias, is intrinsic to the Ig locus and the process of B cell development versus exposure to external antigens has not been determined.

Recently we have demonstrated the functional transplantation of a majority of the human Ig H and kappa L chain loci into the germ line of mice that have been inactivated for endogenous antibody production [22, 23]. These transgenic animals, referred to as XenoMouse™ strains, contain, in substantially germ-line configuration, over 1.0 megabase of the human IgH locus (66 V_H gene segments) and approximately 800 kb of the human Ig kappa locus (32 kappa chain variable gene segments). XenoMouse strains functionally recapitulate the human antibody response including a vast repertoire of high-affinity, somatically hypermutated human antibodies.

As XenoMouse strains contain the majority of the human Ig H and kappa chain loci, essentially in germ-line configuration, we were able to perform experiments on the human humoral response that are not possible in human subjects. For the first time, it was possible to determine

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Abbreviations: RT: Reverse transcription **DIR:** D genes with irregular recombination signals **RSS:** Recombination sequence signals

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the V gene segment utilization of an unimmunized and specific pathogen-free human antibody repertoire. Gene segment utilization of the V, D and J gene segments indicates that XenoMouse strains faithfully reproduce the human adult antibody repertoire.

2 Results

2.1 General remarks

To characterize the utilization of human V gene segments in XenoMouse strains, human antibody transcripts were isolated from adult lymph node and spleen. Following RNA isolation and the generation of cDNA, different V gene-specific oligonucleotides [24] were used in PCR amplification reactions. Mixed primers, representing all the V gene families or primers to a single V gene family were used to generate products that were subsequently cloned. Sequence analysis of the antibody transcripts was performed to identify the relative frequency at which a specific gene segment was found in the repertoire.

Since the XenoMouse strains in this study contain only a single allele of the human H chain locus and a single copy of the human kappa chain locus, these animals are functionally hemizygous. The result is that all H chain transcripts isolated from peripheral secondary immune organs such as the lymph node and spleen represent in-frame functional rearrangements. Although XenoMouse strains have a functional mouse lambda locus, mouse lambda-positive B cells contribute to less than 15 % of the mature B cells and as a result the vast majority of human kappa transcripts (greater than 90 %) represent in-frame and productive rearrangements [23].

2.2 Utilization of V gene segments in XenoMouse human H chain transcripts

The entire human H chain locus that has been introduced into XenoMouse strains is accessible to V(D)J rearrangement. Sequence analysis of the 120 H chain IgM transcripts in this report as well as the sequence analysis of over 50 hybridomas generated from XenoMouse strains (data not shown) has identified 24 of the 34 functional human V_H gene segments present in XenoMouse. There does not appear to be any positional bias in V_H gene segment utilization. V_H segments located both distally, nearly 1 megabase away (4–61), as well as the V_H gene segment most proximal to the J_H locus (6–1) are utilized. The V_H gene segments yet to be detected in XenoMouse H chain transcripts represent the same gene segments that are under-represented in the human repertoire (see below).

Table 1. V_H diversity

	Number utilized/number present in genome[a]	
Family	XenoMouse	Human PBL[b]
V_H1	4/8 (50 %)	6/9 (67 %)
V_H2	1/2 (50 %)	2/3 (67 %)
V_H3	10/15 (67 %)	13/22 (59 %)
V_H4	7/7 (100 %)	7/10 (70 %)
V_H5	1/1 (100 %)	1/2 (50 %)
V_H6	1/1 (100 %)	1/1 (100 %)
V_H7	0	0
Total clones	24/34 (71 %)	30/47 (64 %)

a) Numbers in parentheses indicates percentage of available family members observed in the expressed repertoire.
b) Brezinschek et al. [5].

Similar to H chain transcripts from human PBL, XenoMouse strains show a biased usage of V_H gene segments within a family (Table 1). For example, only 13 of the potentially 22 functional V_H3 family members (59 %) are actually observed in the human peripheral adult human repertoire. In XenoMouse strains only 10 of the 15 functional V_H3 gene segments present on the transgene (67 %) are found expressed in the repertoire. Similarly XenoMouse strains use 4 out of the 8 functional V_H1 family members (50 %), which compares favorably with the 6 out of 9 (67 %) found in human PBL. This pattern was consistent throughout the V_H families and demonstrated that the percentage of functional V_H segments for a given family that is actually present in the XenoMouse repertoire corresponds closely to that reported in humans (Table 1).

The V_H3 and V_H4 families represent the most frequently observed gene segments in the human adult repertoire. To ascertain the frequency of each V_H family in the peripheral repertoire of XenoMouse strains, a human V_H consensus primer was used in conjunction with a C_μ-specific primer to generate PCR products corresponding to human IgM transcripts (see Sect. 4.2). Forty-seven sequences analyzed in this experiment showed a non-random frequency of utilization, with V_H3 and V_H4 families representing 62 % and 25 %, respectively, of the generated transcripts. This is very similar to the 56 % and 20 % frequencies typically found for these V_H families in the human repertoire (Table 2).

Table 2. V_H Family usage[a)]

Family	XenoMouse	Human PBL[b)]
V_H1	1 (2 %)	9 (13 %)
V_H2	0[c)]	3 (4 %)
V_H3	29 (62 %)	40 (56 %)
V_H4	12 (25 %)	14 (20 %)
V_H5	5 (11 %)	4 (6 %)
V_H6	0[c)]	1 (1 %)
V_H7	0	0
Total clones	47	71

a) Numbers in parentheses indicate percentage of all genes detected that were members of each family.
b) Brezinschek et al. [5].
c) Gene segments from the V_H2 and V_H6 family were not observed in the analysis of these 47 sequences but were identified as functional in the analysis of hybridomas and fetal tissues.

Bias in gene segment utilization is even observed within a V_H family; specific V_H gene segments are characteristically over-represented while other gene segments are under-represented in the repertoire of human PBL. For example, V_H gene segments 3–23 and 4–34 represent over a quarter of the observed gene segments derived from the V_H3 and V_H4 families, respectively. To determine if the V_H gene segment utilization in XenoMouse strains is the same as that observed in humans, we examined the utilization of specific V_H gene segments within a V_H family. Reverse transcription (RT)-PCR was performed separately with V_H3 and V_H4 family-specific primers (see Figs. 1 and 2). Seventeen sequences were analyzed using the V_H3 family-specific primer set. As shown in Fig. 1, despite a relatively small sample size, the pattern of V_H segment utilization for the V_H3 family in the XenoMouse repertoire is strikingly similar to that reported for the human repertoire. The frequency of a specific V_H3 gene segment ranges from 0 % to close to 30 %. Notably, the V_H gene segments (3–23, 3–30 and 3–33) that are over-represented in the human peripheral repertoire are also over-represented in the XenoMouse repertoire. In both the human and XenoMouse repertoire V_H3 gene segments 3–20, 3–43, and 3–64 are absent. V_H gene segments 3–13, 3–21, and 3–48 are observed infrequently in both the XenoMouse and human repertoires. Although these latter three V_H3 gene segments were not identified in the set of 17 sequences analyzed in this experiment, they were identified in XenoMouse transcripts from other experiments (data not shown), confirming that they are functional but simply under-represented. A similar analy-

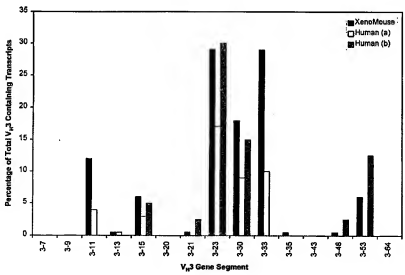

Fig. 1. Relative utilization of V_H3 gene segments. The percentage of specific V_H3 gene segments found in the analysis of 17 XenoMouse-derived transcripts obtained by PCR with a V_H3 family-specific primer is shown. The utilization of V_H3 gene segments in human antibody repertoires is derived from (a) Suzuki et al. [6] and (b) Brezinschek et al. [5]. XenoMouse V_H3 gene segments 3–13, 3–21, 3–35 and 3–48 represented as less than 1 % were not observed in our analysis of 17 V_H3 transcripts but were identified as being functional in other experiments.

sis of 17 sequences generated with a V_H4 family-specific PCR primer set is shown in Fig. 2. A bias in gene segment usage within the V_H4 family was also observed. The same V_H4 gene segments 4-4, 4–31, 4–59 and 4–34 over-represented in XenoMouse strains are also over-represented in the human repertoire (Fig. 2). The V_H4 gene segments 4–28, 4–39, and 4–61 were under-represented in both the XenoMouse and human repertoires.

The results of our analysis of V_H3 and V_H4 gene segment utilization demonstrate that not only are the same V_H gene segments utilized but that they are utilized very much to the same degree. As a result, the V_H gene segment representation in the XenoMouse repertoire appears to be substantially the same as that observed in humans.

2.3 Utilization of D and J_H gene segments in XenoMouse antibody transcripts

In addition to V gene segments, D and J_H gene segment utilization also contributes to the generation of a diverse human H chain repertoire. It is well recognized that the analysis of D segment usage is often difficult because

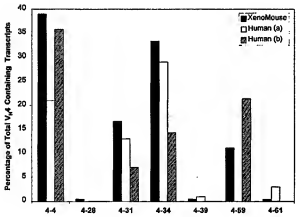

Fig. 2. Relative utilization of V_H4 gene segments. The percentage of specific V_H4 gene segments found in the analysis of 17 XenoMouse-derived transcripts obtained by PCR with a V_H4 family-specific primer is shown. The utilization of V_H4 gene segments in human antibody repertoires is derived from (a) Suzuki et al. [6] and (b) Brezinschek et al. [5]. XenoMouse V_H4 gene segments 4–28, 4–39 and 4–61 represented as less than 1 % were not observed in our analysis of 17 V_H4 transcripts but were identified as being functional in other experiments.

only a small portion of the D segment may be incorporated into the V(D)J junction. Often sequences as short as five nucleotides or less can be identified that have homology to a known D segment. Analysis of 120 XenoMouse transcripts identified 113 with greater than or equal to 5 bases of D sequence homology. Of the 7 transcripts with fewer than 5 bases of homology to a D element, only 2 transcripts could not be assigned to a D segment. Using the nomenclature of Corbett et al. [25] we have identified 19 of the 23 functionally distinct D segments of the human D locus in H chain transcripts derived from XenoMouse strains. The D elements were utilized throughout the D locus and did not show any positional bias in their utilization (Fig. 3). In addition to the conventional D gene segments, 18 % of XenoMouse transcripts had homology that aligned with D genes with irregular recombination signals (DIR) sequences [26].

The J_H gene segment utilization in XenoMouse strains and humans is biased to J_H4 and J_H6. Table 3 shows the frequency of human J_H gene segment usage in 120 XenoMouse-derived H chain transcripts compared to the utilization observed in adult PBL. The most frequently observed J_H segments in the XenoMouse repertoire are J_H4 (46 %) and J_H6 (39 %). Gene segments J_H5 and J_H2 are observed at a frequencies very similar to those observed in the human repertoire, *i.* e. 5 % and 3 %, respectively. The J_H1 utilization in humans is approximately 1 %. A similar frequency in XenoMouse strains may account for the absence of the J_H1 gene segment in the 120 transcripts analyzed. The utilization of J_H gene segments observed in XenoMouse strains closely parallels that reported for human repertoires in every respect (Table 3).

3 Discussion

Our analysis of the human antibody repertoire in XenoMouse strains confirms that the utilization of human Ig V_H gene segments is not random and demonstrates that the human antibody H chain repertoires of XenoMouse strains and human PBL are remarkably similar (Figs. 1 and 2). The precision with which the repertoire of XenoMouse strains has recapitulated the human repertoire is exemplified by the selection of specific V_H3 and specific V_H4 gene family members that are over-represented and under-represented in both the XenoMouse and human repertoires. D segment utilization, including the use of DIR elements unique to primates, D-D fusions and D inversions (data not shown), as well as the preferential utilization of J_H gene segments J_H4 and J_H6 are all observed at frequencies similar to those reported in human repertoires. The human V_κ gene segment utilization in the XenoMouse repertoire is also similar to that

Table 3. Human J_H gene segment utilization

J_H Gene	XenoMouse	Human PBL			
		a)		b)	
J_H1	0 (0 %)	1	(1 %)	1	(1 %)
J_H2	4 (3 %)	3	(4 %)	0	(0 %)
J_H3	9 (7 %)	6	(9 %)	9	(9 %)
J_H4	55 (46 %)	29	(41 %)	52	(52 %)
J_H5	6 (5 %)	5	(7 %)	15	(15 %)
J_H6	47 (39 %	27	(38 %)	22	(22 %)
Total[c]	121	71		99	

a) Brezinschek et al. [5].
b) Yamada et al. [3].
c) Total number of transcripts analyzed.

observed for the human repertoire (data not shown). The $V_\kappa I$ and $V_\kappa III$ families are the most frequently expressed V_κ gene segments and specific V_κ gene segments like A27 and 012 are also abundantly expressed in both the human and the XenoMouse repertoires. The relative utilization of specific $V_\kappa I$ family gene segments in the human kappa repertoire of XenoMouse also parallels that observed in humans.

In conclusion, XenoMouse, with its limited antigenic history, is functioning with apparently the same intrinsic bias in segment utilization as the human repertoire. The basis of the biased usage is still not clear given that V gene segments with identical recombination sequences show dramatic differences in their representation in the human repertoire. The V_H gene segments 4-4 and 4-28 have identical recombination signals [27, 28] yet are observed at dramatically different frequencies in the human repertoire (Fig. 2). DNA sequences flanking the recombination sequence signals (RSS) as well as the roles of enhancers and transcription have all been reported to affect recombination efficiency. Cis-acting elements are also implicitly involved in determining chromatin accessibility that in turn influences targeting of the recombinase to the proper RSS. V(D)J recombination is also very tightly regulated during B cell development; D–J recombination occurs prior to V–DJ recombination that is subsequently followed by recombination of the L chain loci. Recent studies utilizing *In vitro* assays have demonstrated that chromatin accessibility of the recombinase is programmed in different cell lineages [29, 30]. It remains to be determined if V gene segment utilization is also dictated by chromosome accessibility. Other studies have noted differences between individual repertoires and have attributed these differences to gene segment polymorphisms [6, 9, 31]. It remains to be seen whether these polymorphic differences observed in humans result in repertoires that are responsible for autoimmune disease or susceptibility to infection or cancer.

XenoMouse strains represent a significant improvement from earlier transgenic mice carrying a limited number of human Ig genes not only because of the immense size of the loci present in these animals but also because the human loci are in germ-line configuration. As a result, XenoMouse strains contain a human humoral back-

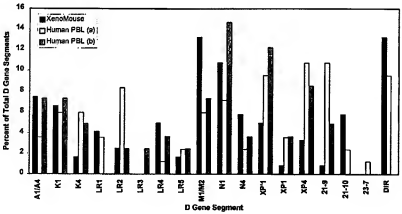

Fig. 3. Usage of D segments in XenoMouse strains and human repertoires. The distribution of D segments in human H chain transcripts based on the sequence analysis of 120 XenoMouse-derived transcripts is shown. The utilization of D elements observed in human antibodies derived from (a) Yamada et al. [3] and (b) Brezinschek et al. [5].

ground and represent a model system that allows for studies not possible in humans. XenoMouse strains can be exploited to determine the significance of the restricted V gene segment utilization that has been observed in human malignancy, autoimmune diseases and responses to bacterial pathogens. They may also be utilized to study V gene segment utilization in response to new vaccines in order to predict their utility in generating the appropriate human immune response. In addition, XenoMouse strains can potentially be used as surrogates for humans to screen humanized antibodies for immunogenicity.

4 Materials and methods

4.1 XenoMouse strains

The generation of XenoMouse strains has been previously described [22, 23]. The XenoMouse strains in this study were functionally hemizygous for the human H chain and human kappa L chain loci. XenoMouse strains are maintained in an SPF and full barrier configured animal facility. Unimmuized mice were used in all experiments.

4.2 RT-PCR and primers

Lymph nodes (approximately 20) and spleens from 4- to 8-week-old XenoMice were isolated and processed according to the manufacturer's instructions using the Micro-Fast Track and Fast-Track 2.0 kits for the isolation of poly (A+) RNA (Invitrogen). The PCR amplification protocol and primers have been previously described [22, 24]. V_H family-specific primers were pooled or used individually as indicated.

4.3 Sequencing and analysis

Sequencing was performed using 4.75 % acrylamide gels, Prism dye terminator sequencing kits and the 373 DNA sequencer (Applied Biosystems). Sequences were analyzed with MacVector and GeneWorks software. The Vbase human antibody database was used for sequence alignments and gene segment identifications (Tomlinson et al., MRC Centre for Protein Engineering). DNA sequences were aligned sequentially to first identify the V_H segment and then the J_H segment. The intervening sequence that had not homology with either the V_H or J_H segments was then aligned against a database of human D segments in V base and the best alignment was identified.

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and bats combined were not significantly different (Fig. 3; echolocators gradient = 0.7932, non-echolocators gradient = 0.8, analysis of covariance (ANCOVA) gradients $F = 0.034$, $p = 0.954$, d.f. 1,20). Thus, it is clear that the estimates of flight cost in echolocating bats are not significantly greater than those for non-echolocating bats.

The absence of an energy cost for echolocation for flying animals may explain why echolocation systems are widespread in the Microchiroptera, but have evolved in very few terrestrial animals. Furthermore the high cost for terrestrial animals[3] may explain why those systems that have evolved in terrestrial mammals involve only very weak short-range pulses[9]. Two alternative hypotheses may also explain the paucity of, and low intensity of, terrestrial echolocation systems. First, the echolocation pulses may reveal the whereabouts of the emitter to potential prey and predators. Alternatively, in a complex and cluttered terrestrial environment the emitter may be confused by strong reflections from very close large objects. Our data strongly support the energy cost hypothesis but cannot rule out these alternative hypotheses in the evolution of echolocation.

The close link between flight and reduced costs for echolocation raises the issues of why echolocation has evolved so infrequently amongst the birds and Megachiroptera, and why, when it has evolved in these animals, it is primarily used for gross navigation rather than prey detection. We suggest that the paucity of echolocation systems amongst these groups reflects a phylogenetic constraint on the development of the processing capacity for complex echolocation signals in animals which are already evolutionarily committed to a visual system. Vision is clearly the dominant system amongst birds. Nocturnal birds have larger cortex areas devoted to processing olfactory stimuli than diurnal birds[10]. As total brain size remains unaffected this suggests there is a trade off in the processing capacity allocated to the various senses. This may have prevented any species from making a complete evolutionary change from one system (visual) to another (echolocation) because the intermediate steps would be selectively inferior to either of the pure systems. The recent suggestion that the megachiroptera have a primate ancestry[11] is consistent with this interpretation because primates also have well-developed vision. □

Received 15 October 1990; accepted 12 February 1991.

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ACKNOWLEDGEMENTS. We thank P. I. Webb and A. M. Burnett for assistance, A. E. Fallick and T. Donnelly of the Scottish Universities Research and Reactor Centre, East Kilbride, for help and advice, and J. P. Hayes, G. C. Hays, M. B. Fenton and J. M. V. Rayner for constructive comments on the manuscript. This work was supported by the NERC.

A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin μ chain gene

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Of the various classes of antibodies that B lymphocytes can produce, class M (IgM) is the first to be expressed on the membrane of the developing cells. Pre-B cells, the precursors of B-lymphocytes, produce the heavy chain of IgM (μ chain), but not light chains[1]. Recent data suggest that pre-B cells express μ chains on the membrane together with the 'surrogate' light chains λ5 and VpreB (refs 2–7). This complex could control pre-B-cell differentiation, in particular the rearrangement of the light-chain genes[8]. We have now assessed the importance of the membrane form of the μ chain in B-cell development by generating mice lacking this chain. We disrupted one of the membrane exons of the gene encoding the μ-chain constant region by gene targeting[9] in mouse embryonic stem cells[10]. From these cells we derived mice heterozygous or homozygous for the mutation. B-cell development in the heterozygous mice seemed to be normal, but in homozygous animals B cells were absent, their development already being arrested at the stage of pre-B-cell maturation.

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The vector used for the disruption of one of the membrane exons (μM) (Fig. 1*A*) contains 9 kilobases (kb) of genomic DNA spanning exons 1 and 2 of μM and the first three exons of the constant (C) region of the δ gene. Close to the 5′ boundary of the first exon of μM we introduced a translational stop codon and a *Sal*I site into which a neomycin-resistance gene (*neo*[r]) cassette[11] was inserted. At the 3′ end of the genomic sequence we placed the herpes simplex virus thymidine kinase gene to permit selection against random integration[12].

Cells of the embryonic stem cell clone D3[13] were transfected with the linearized vector by electroporation and selected by G418 and gancyclovir on feeder layers of STO fibroblasts[14]. Surviving colonies were screened for homologous recombinants using the polymerase chain reaction (PCR) (see legend to Fig. 1). PCR-positive clones were expanded and their identification as homologous recombinants verified by Southern blotting (Fig. 1*B*). From 3.4×10^7 transfected embryonic stem cells, 1,870 colonies were resistant to G418 (determined by control plates), 230 were resistant to both G418 and gancyclovir and in six clones one of the two $C\mu$ genes in the genome was modified by homologous recombination with the vector without random integration. Thus, the frequency of gene targeting was 1/38 $G418^r + GANC^r$ (G418 and gancyclovir-resistant respectively) colonies, which corresponds to 1/312 $G418^r$ colonies or $1/5.7 \times 10^6$ transfected cells.

The mutated clones were injected into blastocysts from C57BL/6 mice to generate chimaeric animals. As the D3 line is derived from an agouti mouse (strain 129/Sv), chimaeric mice could be identified by coat colour. Ten male chimaeras derived from four different mutated clones were mated to C57BL/6 females. One of these chimaeras, derived from clone 210, transmitted embryonic stem-derived chromosomes into the germ line as judged by the production of agouti offspring at a

frequency of 1:6. Three of ten such agouti animals contained the mutated allele as shown by Southern blotting (Fig. 2*b*). These animals permitted a first analysis of the effect of the μM mutation, designated μMT, on B-cell development. Flow cytometric analysis of peripheral blood B cells (identified by the CD45R(B220) surface antigen[15]) demonstrated normal levels of such cells in the heterozygous animals as compared with (C57BL/6 × 129/Sv)F_1 controls, but all cells expressed IgM of C57BL/6 origin, that is of the *b* allotype. This is in contrast to the situation in the F_1 controls where half of the cells express IgM^b and half IgM^a (of 129/Sv origin; Fig. 3). Thus, the μMT mutation is indeed correctly targeted to the $C\mu$ locus, preventing the expression of the membrane form of the μ chain from the targeted allele.

Studies with Abelson virus-transformed pre-B cell lines[8] and immunoglobulin transgenic mice[16] have suggested that the μ chain of membrane-bound IgM (the μ_m chain) plays a crucial role in mediating allelic exclusion, that is, the expression in a single B cell, of only one allele at the heavy-chain loci. The heterozygous mice depicted in Fig. 3 allow this hypothesis to be tested directly: if true, then productive rearrangement of the targeted locus should not preclude productive rearrangement on the other homologue. We indeed found that a small percentage of the B cells in these animals carry a-allotype antibodies of class D on the surface and that low levels of IgM^a can be detected in the serum (data not shown).

The effect of the μMT mutation on the development of B lineage cells can best be studied in homozygous mutant mice. Such animals (14.2 and 14.3; Fig. 2*c*) were generated by intercrossing animals heterozygous for the mutation (G5 end G7; Fig. 2*b*). At the age of 4 weeks, cells from blood, spleen and bone marrow of $\mu MT/\mu MT$ animals were analysed for the presence of B lineage cells by flow cytometry. Cells from heterozygous ($\mu MT/+$) and normal (+/+) littermates served as controls (Fig. 4). As expected, peripheral blood lymphocytes (Fig. 4*a*) and spleen cells (Fig. 4*b*) of the homozygous mutant mice lacked CD45R(B220)^{bright} cells, indicating the absence of mature B cells. We detected neither cells expressing IgM or IgD

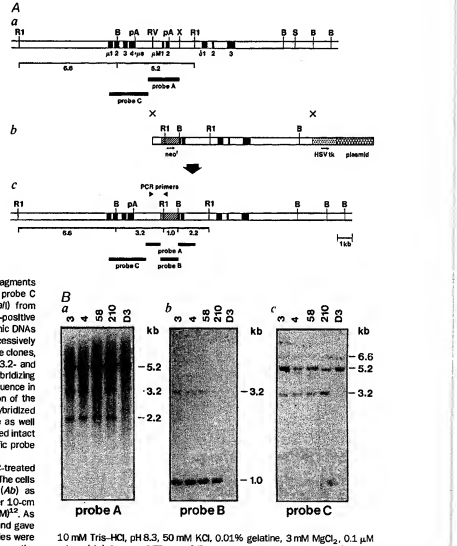

FIG. 1 *A*, Strategy for the disruption of the membrane exon of the $C\mu$ gene. *a*, Genomic structure of the *a* allele of the $C\mu$–$C\delta$ gene locus. Exons are represented by black boxes. pA, polyadenylation sites for the secretory (μ_s) and the membrane (μ_m) form of the μchain[22]. The lengths of diagnostic restriction fragments and location of probes used for Southern blot analysis are shown. RI, *Eco*RI; B, *Bam*HI; RV, *Eco*RV; X, *Xho*I, S. *Sal*I; *b*, Targeting vector containing a 9-kb *Eco*RV–*Sal*I fragment of the $C\mu$–$C\delta$ locus. The third codon of membrane exon 1 ($\mu M1$ in *a*) was changed to TAG followed by an insertion of TCGAC to create a *Sal*I site by site directed mutagenesis. Five base pairs (bp) downstream of the *Sal*I site, 7 bp were accidentally deleted. An *Xho*I–*Sal*I fragment (*neo*^{r}) of pMC1Neo-polyA[14](PMC1POLA, STRATAGENE) was inserted into the new *Sal*I site. The *Eco*RV–*Sal*I fragment carrying the *neo*^{r} gene was inserted into an *Xho*I site of pIC19R/MC1–TK[12] (gift of K. Thomas, S. Mansour and M. Capecchi) which contains the HSV *tk* gene. The vector was linearized by *Cla*I before transfection. *c*, Predicted structure of the targeted locus. Triangles indicate the primers used for PCR assays. The sequence of the 5′ primer (5′-CTCTGTAAC-CACTCACCACC-3′) is located 96 bp upstream of the *Eco*RV site shown in *a*. The sequence of the 3′ primer (5′-CCTGC-GTGCAATCCATCTTG-3′) is located 320 bp downstream of the *Xho*I site in pMC1Neo-polyA. The lengths of diagnostic restriction fragments and hybridization probes are indicated. Probe A (*Nco*I–*Xho*I) and probe C (*Nco*I–*Nco*I) are derived from the $C\mu$ locus, probe B (*Xho*I–*Sal*I) from pMC1Neo-polyA. *B*, Southern blot analysis of D3 cells and PCR-positive transfectants (four clones—3, 4, 58 and 210—are shown). Genomic DNAs were digested by *Bam*HI and *Eco*RI. The filter was hybridized successively by the three probes indicated below each panel. *a*, In the PCR-positive clones, probe A hybridized to a wild-type 5.2-kb fragment as well as to 3.2- and 2.2-kb fragments in the targeted locus, as expected. *b*, Probe B hybridizing to 3.2- and 1.0-kb fragments shows the presence of the *neo*^{r} sequence in the locus. The faint 4.2-kb band is probably due to partial digestion of the DNA. *c*, As predicted, probe C, located outside the targeting vector, hybridized in the PCR-positive clones to the same 3.2-kb fragment as above as well as to the wild type fragments (6.6 and 5.2 kb). The *C8* locus remained intact in the mutated allele as shown by hybridization with a *C8*-specific probe (data not shown).

METHODS. D3 cells[13] (gift of E. Wagner) were grown on mitomycin C-treated G418-resistant STO fibroblasts[14] (gift of C. Kappen and F. Ruddle). The cells were electroporated with 20 $\mu g\ ml^{-1}$ of the linearized vector (*Ab*) as described[21]. The cells were plated at a density of 6×10^6 cells per 10-cm feeder plate and selected with G418 (200 $\mu g\ ml^{-1}$) and GANC (2 μM)[12]. As a control, 2×10^6 transfected cells were selected in G418 only, and gave rise to 110 G418^r colonies. After 11–14 days, G418^r–GANC^r colonies were screened for homologous recombination by PCR analysis as follows: the colonies were picked and trypsinized in multi-well plates individually. Half of the cells from each colony were cultured on multi-well plates. The other cells (8-12 colonies) were pooled for PCR analysis. Pooled samples were treated with proteinase K (ref. 23) and gene amplification was done in 50 μl 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 0.01% gelatine, 3 mM $MgCl_2$, 0.1 μM primer (*Ac*), 0.4 mM dNTPs and 2.5 U *Taq* polymerase for 40 cycles (1.5 min at 95 °C, 1 min at 65 °C and 2 min at 72 °C). Samples containing targeted cells gave a 1-kb amplified fragment. The clones from PCR-positive pools were then analysed individually and positive clones were expanded and analysed by Southern blotting (*B*).

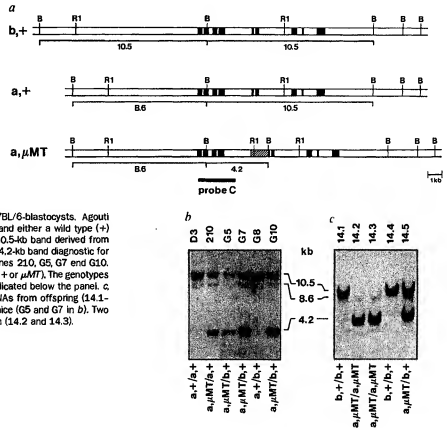

FIG. 2 *a*, Genomic structure of the $C\mu$-$C\delta$ loci of the *a* and *b* alleles and of the μMT mutant with predicted sizes of the fragments detected by probe C in Southern blot analysis (*b* and *c*). The Igh^b wild type allele (b_1+; top) is from C57BL/6. The Igh^a wild type (a,+; middle) and mutated ($a_1\mu MT$; bottom) alleles are from the mutated ES clones. *b*, Southern blot analysis showing germ-line transmission of the μMT mutation. DNAs from D3 cells (D3), a mutated D3-clone (210, Fig. 1*B*) and tails of agouti offspring (G5, G7, G8 and G10) were digested by *Bam*HI and hybridized with probe C. The offspring was from a C57BL/6 female mated to a chimaeric male which had been generated by the injection of clone 210 into C57BL/6-blastocysts. Agouti offspring carry an Igh^b allele of C57BL/6 origin and either a wild type (+) or a mutated (μMT) Igh^a allele of D3 origin. A 10.5-kb band derived from Igh^b or wild type Igh^a was present in all lanes. A 4.2-kb band diagnostic for the mutated allele (Igh^a, μMT) was present in lanes 210, G5, G7 and G10. A weak 8.6-kb band is derived from the Igh^a allele (+ or μMT). The genotypes of the $C\mu$ loci determined by the analysis are indicated below the panel. *c*, Southern blot analysis of *Bam*HI-digested tail-DNAs from offspring (14.1–14.5) of an intercross of heterozygous mutant mice (G5 and G7 in *b*). Two animals were homozygous for the μMT mutation (14.2 and 14.3).

on the surface nor B cells in the peritoneal cavity (data not shown). In addition, the homozygous mutant mice had no detectable IgM in the serum (<0.1 $\mu g\,ml^{-1}$), compared with ~600 μg IgM per ml in the controls (data not shown). In contrast, $\mu MT/\mu MT$ mice generate substantial numbers of T cells, as demonstrated by staining with anti-Thy-1 (Fig. 4*a*) or anti-CD3 (Fig. 4*b*) antibodies.

Which stage of B cell development is affected by the μMT mutation? B cells are continuously generated from stem cells in the bone marrow. The latter cells first differentiate into pre-B cells, detectable by flow cytometry as $CD45R(B220)^{dull}$, surface $IgM^-(sIgM^-)$ cells[17]. Through rearrangement of the L chain genes, the pre-B cells give rise to $CD45R(B220)^{dull}$, $sIgM^+$ B cells[1,17]. In the bone marrow of the homozygous mutant mice $sIgM^+$ cells were absent (Fig. 4*c*). By contrast, the frequency of cells of the pre-B cell phenotype was roughly the same as in the controls, although the level of CD45R(B220) expression in these cells seemed slightly lower. Pre-B cells go through an early phase of proliferation as large cells from which small, non-dividing pre-B cells originate[18–20]. Analysis of the $CD45R(B220)^{dull}$ cells in the homozygous mutant mice by forward light scatter showed that they were mainly large, in contrast

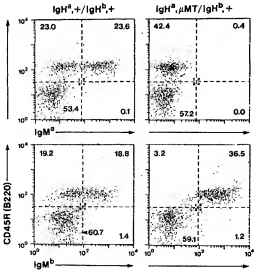

FIG. 3 Flow cytometric analysis (FACScan, Becton–Dickinson) of peripheral blood lymphocytes (PBL) from 3-month-old F_1 mice carrying (Igh^a, $\mu MT/Igh^b$, +; mouse G7 in Fig. 2*b*) or not carrying (Igh^a, +/Igh^b, +, mouse G8 in Fig. 2*b*) the μMT mutation. Peripheral blood lymphocytes were purified on a Ficoll-gradient and stained with phycoerythrin (PE)-conjugated monoclonal antibody RA3-6B2[24] (anti-CD45R(B220)) and fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies, RS3.1[25] (anti-IgM^a) or MB86[26] (anti-IgM^b). Dots and percentages in the fluorescence windows refer to cells in the lymphocyte gate as defined by light scatter[17]. The vertical and horizontal axes show intensity of red (PE) and green (FITC) fluorescence, respectively.

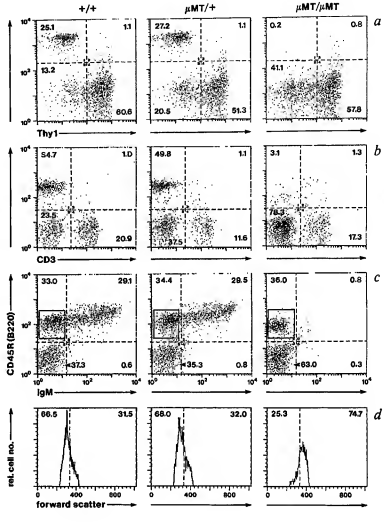

FIG. 4 Flow cytometric analysis of lymphocytes from homozygous mutant ($\mu MT/\mu MT$; 14.3 in Fig. 2c), normal (+/+; 14.4 in Fig. 2c) and heterozygous mutant ($\mu MT/+$; 14.5 in Fig. 2c) littermates. *a*, PBLs. *b*, spleen cells or *c*, bone marrow cells from 4-week-old mice were stained with monoclonal antibody RA3-3A1[15]-PE (anti-CD45R(B220)), and mAbs CFO 1[27]-FITC (anti-Thy 1, *a*) or 145-2C11[28]-FITC (anti-CD3, *b*) or R33-24-12[29] FITC (anti-IgM, *c*). Dead cells were excluded by propidium-iodide staining in *b* and *c* (See also legend of Fig. 3.) The boxes in *c* define those cell populations reanalysed for cell size by forward light scatter as displayed in *d* in the form of histograms.

to the pre-B cells in controls, of which only a small proportion were large (Fig. 4*d*). Therefore, the μMT mutation seems to arrest B-cell differentiation at the pre-B cell stage, presumably close to, or at the point of, transition from large to small pre-B cell. But, it cannot be excluded that the CD45R(B220)^{dull} cells in the bone marrow of the homozygous mutant mice do not represent B-lineage cells; in this case the μMT mutation would prevent pre-B cell generation altogether.

The present work established that B-cell development is dependent on the expression of the membrane form of the μ chain by the pre-B cell stage. This implies a function for this protein before L chain expression and supports the idea that μ_m together with 'surrogate' L chains forms a membrane receptor through which pre-B-cell differentiation is controlled. Furthermore, the μMT mouse mutant both allows testing of the hypothesis that allelic exclusion is mediated through μ_m expression and offers itself as a model of an immunodeficiency selectively affecting B-cell development, as occurs in humans[21]. □

Received 14 January; accepted 1 February 1991.

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ACKNOWLEDGEMENTS. We thank W. Müller for reagents and advice, U. Ringelsen for artwork and E. Siegmund and G. Schmall for typing the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft through SFB 243 and the Fazit Foundation. D. Kitamura was supported by a fellowship from the Alexander von Humboldt Foundation. J. Roes from the Fonds der Chemie.